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## Original Research Article

# *Allium tuberosum* alleviates pulmonary inflammation by inhibiting activation of innate lymphoid cells and modulating intestinal microbiota in asthmatic mice



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## ABSTRACT

**Objective:** This study tests whether long-term intake of *Allium tuberosum* (AT) can alleviate pulmonary inflammation in ovalbumin (OVA)-induced asthmatic mice and evaluates its effect on the intestinal microbiota and innate lymphoid cells (ILCs).

**Methods:** BALB/c mice were divided into three groups: phosphate buffer saline, OVA and OVA + AT. The asthmatic murine model was established by sensitization and challenge of OVA in the OVA and OVA + AT groups. AT was given to the OVA + AT group by oral gavage from day 0 to day 27. On day 28, mice were sacrificed. Histopathological evaluation of lung tissue was performed using hematoxylin and eosin, and periodic acid-Schiff staining. The levels of IgE in serum, interleukin-5 (IL-5) and IL-13 from bronchoalveolar lavage fluid (BALF) were measured by enzyme-linked immunosorbent assay. The ILCs from the lung and gut were detected by flow cytometry. 16S ribosomal DNA sequencing was used to analyze the differences in colon microbiota among treatment groups.

**Results:** We found that long-term intake of AT decreased the number of inflammatory cells from BALF, reduced the levels of IL-5 and IL-13 in BALF, and IgE level in serum, and rescued pulmonary histopathology with less mucus secretion in asthmatic mice. 16S ribosomal DNA sequencing results showed that AT strongly affected the colonic bacteria community structure in asthmatic mice, although it had no significant effect on the abundance and diversity of the microbiota. *Ruminococcaceae* and *Desulfovibrionaceae* were identified as two biomarkers of the treatment effect of AT. Moreover, AT decreased the numbers of ILCs in both the lung and gut of asthmatic mice.

**Conclusion:** The results indicate that AT inhibits pulmonary inflammation, possibly by impeding the activation of ILCs and adjusting the homeostasis of gut microbiota in asthmatic mice.

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## 1. Introduction

The prevalence of asthma has increased by almost 30% in the past two decades, and millions of patients are suffering from it each year [1,2]. It is a challenge to improve the life quality of individuals suffering from asthma. As we all know, food plays an essential role in maintaining health [3]. It is accepted that diet plays an important role in some diseases, such as type 2 diabetes,

cardiovascular diseases and cancers [4,5], and it also has been noted that food can play a vital role in allergic asthma [6,7].

In China, *Allium tuberosum* (AT, also called garlic chives) is used for not only culinary purposes but also treating abdominal pain, diarrhea, diabetes and chronic diseases, due to its medicinal value. Recent studies have proved the anticancer, antioxidant, aphrodisiac, anti-inflammatory and nematocidal properties of AT, as well as its treatment effects on diabetes and hepatitis; a variety of substances extracted from AT show strong anti-inflammatory potential [8–13]. Asthma is mainly characterized by type 2 inflammation. Drugs that relieve pulmonary inflammation can provide effective asthma treatment, suggesting that AT might be able to

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treat asthma by reducing pulmonary inflammation. However, the impact of AT on asthma, intestinal microbiota and the innate immune system has never been reported.

Innate lymphoid cells (ILCs) are innate counterparts of adaptive T lymphocytes and were identified less than a decade ago. They contribute to host defence, metabolic homeostasis and combatting inflammatory diseases [14]. Unlike the role of T helper (Th) cells, ILCs can also regulate the nonallergic inflammation of asthma [15]. ILCs have four subgroups, ILC1, ILC2, ILC3 and ILCreg, whose functions are similar to the four subtypes of Th cells. ILC2, found mainly on the mucosal surface, is the most prevalent ILC subtype in the lungs, at steady state [16]. The proportion of ILC2 increased significantly in lung tissue from asthmatic mice, contributing to asthmatic attacks by rapidly secreting IL-5 and IL-13, with the result of aggravating lung eosinophilia, mucus production and airway hyperresponsiveness [14,17]. ILCs from the intestine also play an important role in shaping and maintaining the stability of the human microbiota [18–20].

Emerging evidence relates asthma with the composition and function of gut microbiota. It has been shown that the intestinal microbiota can affect maturation of the immune system and enhance risk of allergic asthma in experimental models and clinical studies [21,22]. Studies have shown that the decrease in diversity of early intestinal microbiota is closely related to the asthmatic phenotype in children, while the decrease in *Bifidobacterium* and the increase in *Clostridium difficile* have also been observed in the gut of these patients [23]. It also has been shown that treating 3-week-old mice with antibiotics reduces the diversity of their microbiota, thus aggravating allergic airway inflammation induced by airway allergens [24]. However, probiotics can play a protective role in asthma, and loss of gut microbial diversity could lead to the exacerbation of asthma [25]. Finally, the diversity of early intestinal microbiota groups also plays an important role in the development of the immune system [20].

These findings indicate that both gut microbiota and ILCs play essential roles in the development of asthma diseases. Therefore, in this study we test whether AT can improve the asthmatic phenotype, and evaluate how AT shapes the intestinal microbiota and ILCs in asthmatic mice.

## 2. Materials and methods

### 2.1. Animals

Female BALB/c mice, six to eight weeks old, were purchased from Beijing Vital River Laboratory Animal Technology Company™ (Beijing, China). They were housed in a pathogen-free facility with a 12-h light/dark cycle and given free access to food and water. All animal studies were approved by the Animal Ethics Committee of the Beijing University of Chinese Medicine (BUCM-4-2018120601-4054). Animal experiments followed the guidelines of the Beijing University of Chinese Medicine Animal Care and Use Committee.

### 2.2. Preparation of AT food mixture

AT was purchased from JD.com (Beijing, China). After washing, 500 g of AT was boiled for 3 h, then the AT and the water it cooked in were homogenized and diluted with sterile water to a final volume of 1000 mL. Food mixture at a concentration of 0.5 g/mL was given to a mouse (0.2 mL) per day, according to 40 g daily intake of AT for humans.

### 2.3. Establishment of allergic asthma

Mouse allergic asthma was induced as described by Xu et al. [26] with some modification. After one week for acclimatization,

BALB/c mice were randomly divided into three groups, with at least six mice per group as follows: phosphate buffer saline (PBS) group, ovalbumin (OVA) group and OVA + AT group. The mice in the OVA group and OVA + AT group were sensitized on days 0 and 14 by intraperitoneal injection with 20 µg of OVA (purchased from Sigma-Aldrich™, St. Louis, MO, USA) in PBS, mixed with a half volume of alum as an adjuvant in a total volume of 200 µL. From day 0 to day 27, the mixture of AT was given to the OVA + AT group by oral gavage, while normal saline was given to the PBS and OVA groups. On days 21–27, the mice of the OVA and OVA + AT groups were exposed to aerosolized OVA (1% OVA in PBS) for 30 min per day, while the mice of the PBS group were given PBS alone (Fig. 1).

### 2.4. Serum and bronchoalveolar fluid collection, and cell counting

On day 28, mice were anesthetized with isoflurane prior to collecting a blood sample and then euthanized by cervical dislocation. Serum was obtained by centrifuging the blood at 4500×g for 15 min at 4 °C, and it was stored at –80 °C until it was analyzed. The lungs of mice were lavaged three times with 1 mL of normal saline with 1% fetal bovine serum (Gibco), and the fluid was retained. This bronchoalveolar lavage fluid (BALF) was centrifuged (1500×g) immediately for 5 min at 4 °C, and the supernatant was stored at –80 °C for cytokine detection. The pellet was resuspended in PBS and stained with trypan blue. The total number of cells was determined under a microscope.

### 2.5. Histological examination of lung tissue

Histological examination of lung tissue was used to further assess disease progression. Lungs were washed in PBS, fixed in 10% formaldehyde at room temperature, dehydrated in a concentration gradient of ethanol and embedded in paraffin. Four-micrometer thick tissue sections were stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) to evaluate the general morphology [27].

### 2.6. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay kits were used to assess levels of IgE (Biolegend™, 431401) in serum, and IL-5 (Biolegend, 79077) and IL-13 (ebioscience™, 4290085) in BALF, following the manufacture's instructions.

### 2.7. Cell isolation from lung and gut tissues in mice

Single-cell suspension was prepared according to the methods of Bando and Colonna [19]. In brief, mice were anesthetized with isoflurane and euthanized by cervical dislocation on day 28. To isolate the lung tissue cells, lungs were minced and incubated with 1 mg/mL collagenase IV (Worthington™, LS004183) and 50 µg/mL DNase I (Roche™, 11284932001) in RPMI-1640 media, for 40 min at 37 °C, before being mashed through 70 µm cell strainers. For isolation of cells from the intestine, after adherent fat tissue and Peyer's patches were removed, the gut was opened

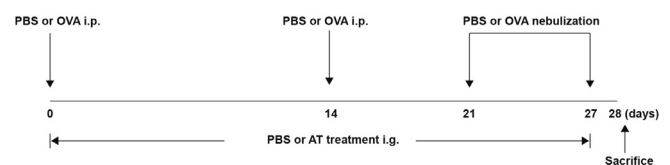


**Fig. 1.** A schematic view for the induction of allergic asthma along with AT treatment in mice. PBS: phosphate buffer saline; OVA: ovalbumin; i.p.: intraperitoneal; AT: *Allium tuberosum*.

longitudinally, and cut into 5 mm pieces. Pieces were washed twice with 20 mL Hank's Balanced Salt Solution medium containing 5 mmol/L ethylene diamine tetraacetic acid and 1 mmol/L dithiothreitol for 20 min at 37 °C, to remove epithelial cells. Then, gut tissues were digested with 2 mg/mL collagenase III (Worthington™, LS004183) and 50 µg/mL DNase I in RPMI-1640 media for 40 min. Single-cell suspensions from both tissues were used for subsequent flow cytometry staining.

## 2.8. Flow cytometry analysis

Single-cell suspensions were kept at 4 °C, and nonspecific binding was blocked by anti-Fc receptor-blocking antibody (anti-CD16/32), before cell surfaces were stained with biotinylated monoclonal antibodies specific for CD3ε, CD4, CD8α, CD11c, CD11b, CD19, TCRβ, TCRγδ, NK1.1, Gr1, Ter119 and B220. Then cells were stained with Alexa Fluor®488-conjugated streptavidin, APC-Cy7-conjugated anti-mouse CD45, PE-Cy7-conjugated anti-mouse CD90.2 and APC-conjugated anti-mouse Sca1. Cells were detected by FACSCanto™ (BD, Biosciences) and analyzed using Diva software.

## 2.9. 16S ribosomal DNA gene microbiome analysis

DNA samples taken from the feces of BALB/c mice in different groups were subjected to 16S rRNA gene sequence-based analysis, to examine the specific bacterial communities along the mouse intestinal tract. Small fragment libraries, whose concentrations were more than 30 ng/µL, were used for polymerase chain reaction (PCR) amplification. The samples were detected before treatment, after being thawed on ice, centrifuged, and mixed thoroughly. Qubit test was used to test the sample concentration. The components and volumes of the reagents in the PCR amplification system included DNA sample X µL (30 ng), forward primer (10 µmol/L) 2 µL, reverse primer (10 µmol/L) 2 µL, dNTPs (2.5 mmol/L) 4 µL, 10 × Pyrobest buffer 5 µL, Pyrobest DNA polymerase 3 µL, and ddH<sub>2</sub>O (34–X) µL (total 50 µL). The PCR procedure condition was setting at 95 °C for 5 min and then 95 °C for 30 s and then 56 °C for 30 s, 25 cycles and then 72 °C for 40 s and then 72 °C for 10 min, finally was ending in 4 °C. Products were examined on a 2% (150 V, 30 min) agarose gel. The primers were 338F (5'-ACTCC TACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTC TAAT-3'). All procedures followed the manufacture's protocol (Allwegene Company™, Beijing, China). Two hypervariable regions of 16S rRNA, the V3 and V4, can be used to identify the vast majority of bacteria through 16S rRNA sequencing. The Illumina HiSeq 2000 platform was used to sequence the V3 and V4 regions through positive and negative reading. Only sequences longer than 50 bp were used for phylotype analysis at the 0.03 operational taxonomic unit (OTU) level. Bioinformatics analysis was carried on with sequencing data.

## 2.10. Statistical analysis

Results are presented as mean ± standard error of the mean. One-way analysis of variance was used for multiple comparisons, followed by the Mann-Whitney test, where further nonparametric analysis was appropriate. A *P*-value of less than 0.05 was considered statistically significant.

## 3. Results

### 3.1. Effects of AT on the airway inflammation of OVA-induced asthmatic mice

In our study, OVA-induced asthmatic mice showed a high level of infiltration of pulmonary inflammatory cells and a high level of

total IgE in serum. Compared with the PBS group, the OVA group developed severe perivascular and bronchial inflammation. This manifested in the infiltration of leukocytes and mucus secreted around the bronchus, the thickening of the alveolar wall and the narrowing of the alveolar cavity. Moreover, the secretion of mucus and the inflammatory infiltration were less pronounced in the OVA + AT group, compared with the OVA group (Fig. 2A). Cells from BALF were collected at the end of the experiment, and total inflammatory cell numbers in the OVA + AT group were significantly decreased compared with the OVA group (*P* < 0.05; Fig. 2B). A significant increase of IgE concentration was observed in the OVA group when compared with the PBS group (*P* < 0.001), while the OVA + AT group showed lower IgE concentration compared with the OVA group (*P* < 0.05; Fig. 2C). Further, a significant reduction of IL-5 and IL-13 concentrations in BALF was detected in the OVA + AT group, compared with the OVA group (*P* < 0.01; Fig. 2D and E).

### 3.2. Effects of AT on the numbers of ILCs from the lung and intestine in OVA-induced asthmatic mice

Consistent with the previous study, ILC2 contributes to allergic inflammation by secreting type 2 cytokines such as IL-4, IL-5 and IL-13 and recruiting other inflammatory cells [28]. Moreover, it has been shown that ILC2 has the ability to direct cells to distant organs participating in different immune responses [29]. Changes in levels of ILCs in various tissues are related to disease conditions, so we tested the absolute number of ILCs in different tissues. Here the CD45<sup>+</sup>Lin<sup>−</sup>CD90.2<sup>+</sup>Sca1<sup>+</sup> cells were considered to be ILCs [30]. It showed that the total numbers of ILCs from these three tissues in the OVA group increased significantly compared to the PBS group, while OVA + AT treatment suppressed those augmentations in asthmatic mice (Fig. 3A and B). The increased ILCs in these mucosal tissues might be the main producers of the inflammatory cytokines that caused the pulmonary infiltration in asthmatic mice. The amelioration of AT on improvement of inflammation in exudates of asthmatic mice treated with AT may be related to the reduction in numbers of ILCs.

### 3.3. Effects of AT on the richness and diversity of intestinal microbiota in OVA-induced asthmatic mice

To further investigate the mechanism of AT on changes in asthmatic mice, gut bacterial richness and diversity were analyzed using 16S ribosomal DNA sequencing. In total, after size filtering, quality controlling and removal of chimera, we obtained 1,044,017 valid sequences, with an average of 69,601 sequences from each sample. Statistical analysis of sequence data at a 97% of similarity level resulted in a total of 487 OTUs, and the average OTU number of the PBS, OVA and OVA + AT groups were 215, 304 and 303, respectively. All samples reached a stable plateau based on rarefaction curve analysis, indicating the sampling was sufficient for the majority of the bacterial communities.

It showed that, compared with the PBS group, both the Chao-1 and Shannon indexes of total intestinal microbiota from the OVA group increased significantly, which indicates the increase of abundance and diversity. However, compared with the OVA group, the Chao-1 and Shannon indexes of the OVA + AT group showed no significance (Table 1; Fig. 4A). Therefore, we further analyzed the difference in composition of the intestinal flora using unifracs-based principal coordinate analysis (PCoA) and analysis of similarities (ANOSIM). PCoA analysis suggests that there were differences among the control, model and OVA + AT groups. In further ANOSIM analysis, we found that all pairwise comparisons showed statistical difference (*P* < 0.05). This indicates that the intestinal bacteria populations of each of the three groups were distinct (Fig. 4B and C).

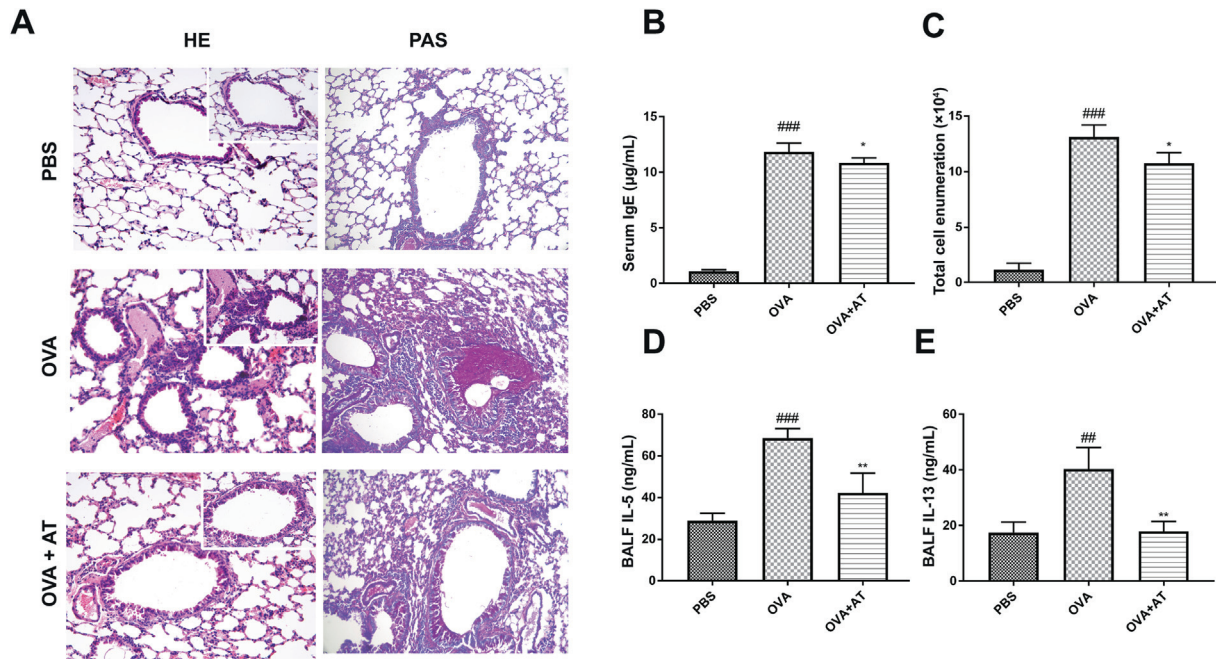


**Fig. 2.** AT mitigated the pulmonary inflammation in the OVA-induced asthmatic model. A: Effects of AT on lung tissue histology in asthmatic mice (×20 and ×40 for HE staining; ×20 for PAS staining). Cellular infiltration was observed in HE staining and mucus secreted in PAS staining. B: Levels of serum total IgE from mice. C: Numbers of total leukocytes in BALF. D: Levels of IL-5 in BALF. E: Levels of IL-13 in BALF. All data are expressed as mean ± standard error of mean. <sup>###</sup>*P* < 0.01, <sup>###</sup>*P* < 0.001, compared with PBS group; <sup>\*</sup>*P* < 0.05, <sup>\*\*</sup>*P* < 0.01, compared with OVA group. *n* = 6 per group. AT: *Allium tuberosum*; OVA: ovalbumin; HE: hematoxylin and eosin; PAS: periodic acid-Schiff; PBS: phosphate buffer saline; BALF: bronchoalveolar lavage fluid; IL: interleukin.

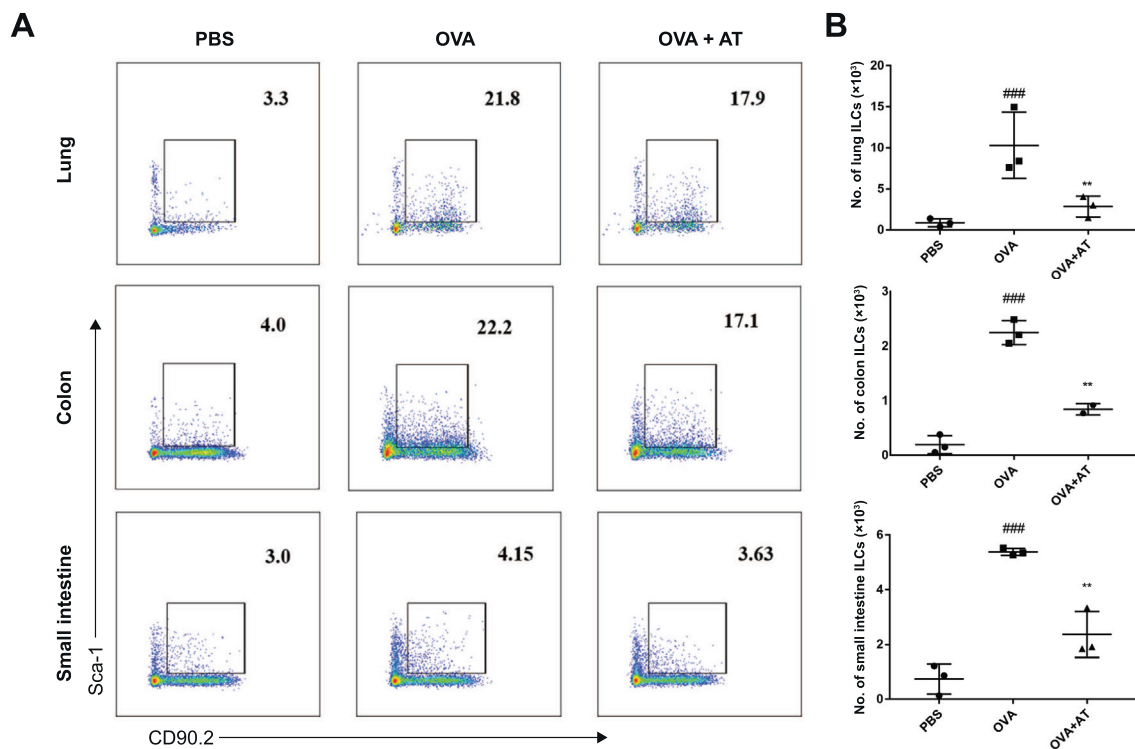


**Fig. 3.** Effects of AT on the numbers of ILCs from the lung and intestine in OVA-induced asthmatic mice. A: Flow cytometric profiles of ILCs from the lung, small intestine and colon in three groups at the end of experiments, gated on lineage-CD45<sup>+</sup>CD90.2<sup>+</sup>Sca-1<sup>+</sup>. Numbers indicate the frequency of flow cytometric events. B: Absolute numbers of ILCs in the lung, small intestine and colon. All data are expressed as mean ± standard error of mean. <sup>###</sup>*P* < 0.001, compared with PBS group; <sup>\*\*</sup>*P* < 0.01, compared with OVA group. *n* = 3 per group. AT: *Allium tuberosum*; OVA: ovalbumin; ILC: innate lymphoid cell; PBS: phosphate buffer saline.

**Table 1**  
Effects of AT on bacterial communities' diversity in the colon from mice.

| Sample ID  | Valid sequence | Similarity score $\geq 0.97^1$ |        |         |
|------------|----------------|--------------------------------|--------|---------|
|            |                | OTU                            | Chao-1 | Shannon |
| PBS 1      | 40,356         | 265                            | 297.1  | 5.91    |
| PBS 2      | 41,254         | 222                            | 255.3  | 5.23    |
| PBS 3      | 28,567         | 184                            | 228.4  | 4.24    |
| PBS 4      | 42,951         | 203                            | 241.7  | 4.51    |
| PBS 5      | 22,416         | 202                            | 223.2  | 4.46    |
| OVA 1      | 76,729         | 339                            | 362.7  | 6.53    |
| OVA 2      | 81,123         | 314                            | 344.6  | 6.03    |
| OVA 3      | 98,198         | 303                            | 348.2  | 5.65    |
| OVA 4      | 80,189         | 303                            | 345.3  | 6.04    |
| OVA 5      | 41,872         | 261                            | 296.6  | 5.27    |
| OVA + AT 1 | 113,195        | 289                            | 308.7  | 6.31    |
| OVA + AT 2 | 122,911        | 279                            | 358.0  | 5.70    |
| OVA + AT 3 | 138,874        | 328                            | 376.5  | 6.18    |
| OVA + AT 4 | 70,281         | 307                            | 354.5  | 6.10    |
| OVA + AT 5 | 45,101         | 310                            | 373.7  | 5.48    |

<sup>1</sup> Sequences with similarity scores greater than or equal to 0.97 were clustered into an operational taxonomic unit (OTU). PBS: phosphate buffer saline; OVA: ovalbumin; AT: *Allium tuberosum*.

### 3.4. Effects of AT on colonic bacteria community structure in OVA-induced asthmatic mice

The colonic bacterial community was further analyzed to reveal the role of AT on the homeostasis of gut microbiota. All sequences were classified and identified from phylum to genus. We found that there were 280 shared OTUs among three groups, accounting for 57.4% of the total OTUs (Fig. 5A). At the phylum level, *Firmicutes* and *Bacteroides* accounted for more than 95% of the total flora, which are the dominant flora. In the OVA + AT group, the *Firmicutes* populations had increased to 50%, compared to the 34% in the OVA group ( $P < 0.05$ ). At the same time, the *Bacteroides* population in the OVA + AT group decreased to 42% from 63% in the OVA group ( $P < 0.05$ ; Fig. 5B). At the genus level, a total of 105 genera were detected. We found a total of 90 flora, 15 of which had a relative abundance greater than 0.1. According to mean relative abundance and  $P$ -value ( $P < 0.05$ , relative abundance  $> 0.1\%$ ), we found 14 eligible genera (Fig. 5C).

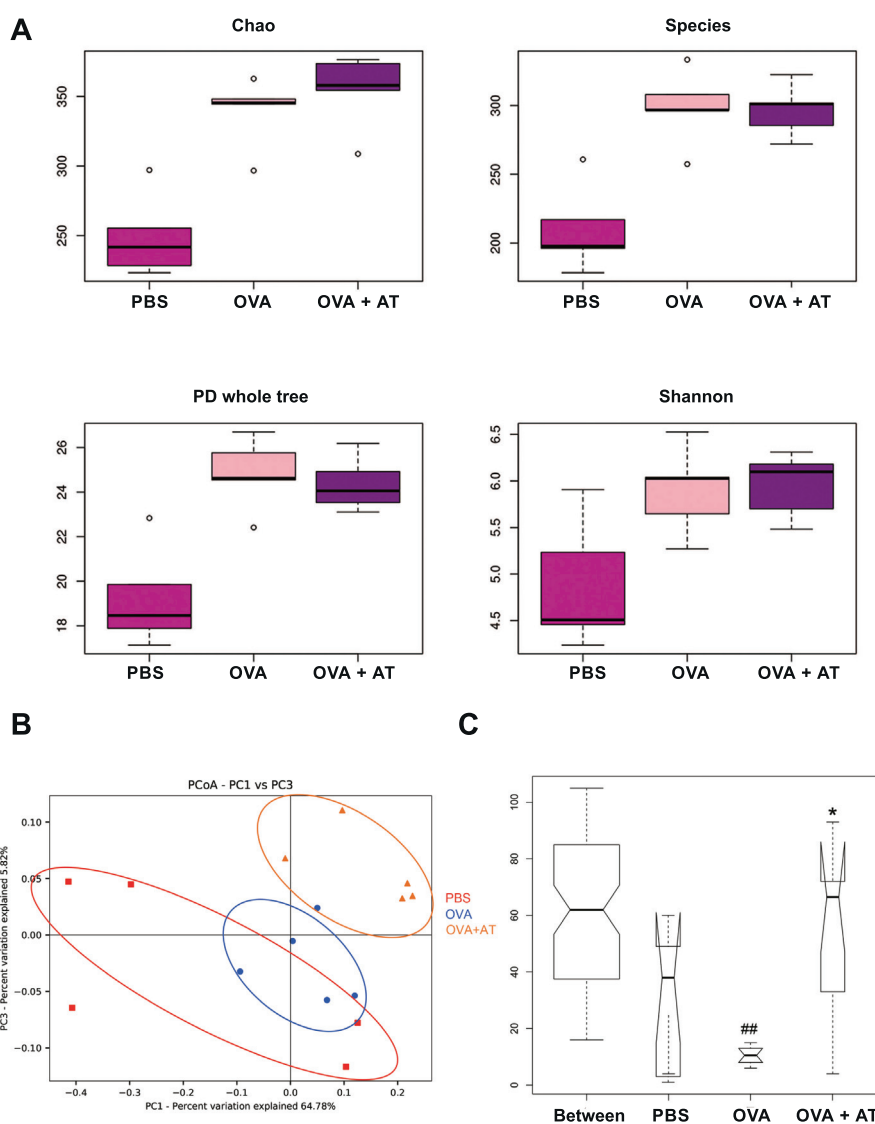


**Fig. 4.** Community difference analysis of three groups. A: Comparison of alpha diversity of Chao index, observed species, PD whole tree and Shannon index among three groups. B: Principal coordinate analysis (PCoA) on weighted unifrac distances of the three groups. C: ANOSIM analysis. "Between" represents the difference among the three groups; the closer the R-value is to 1, the greater the difference among the groups. ## $P < 0.01$ , compared with the PBS group; \* $P < 0.05$ , compared with the OVA group. "PBS" "OVA" and "OVA + AT" represent the different three groups.  $n = 5$  per group. PBS: phosphate buffer saline; OVA: ovalbumin; AT: *Allium tuberosum*.

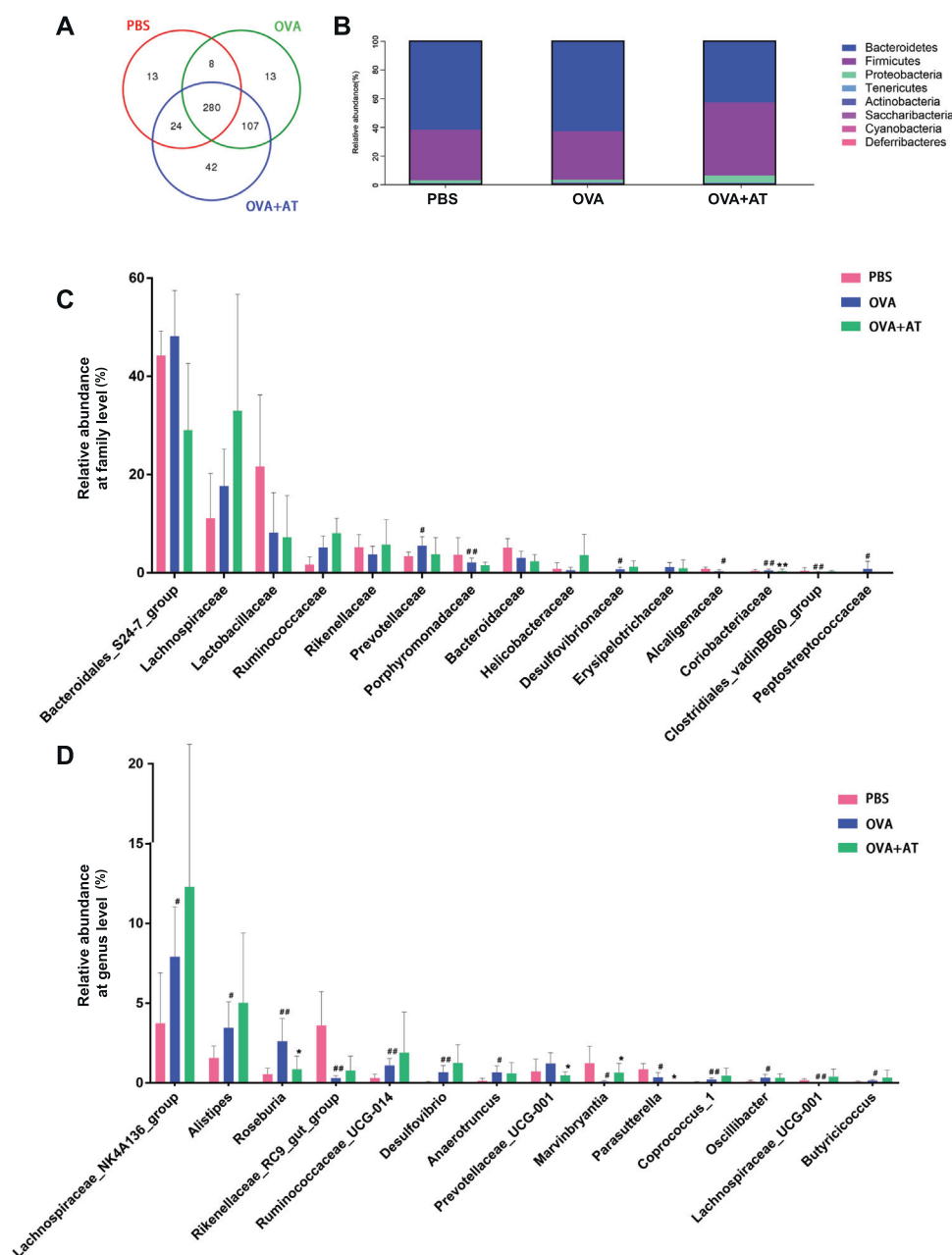


**Fig. 5.** Comparison of microbial composition from three groups. A: Venn diagram of the shared and unique OTUs among three groups. B: Relative abundance of three groups at the phylum level. C and D: Relative bacterial abundance of three groups at the family (C) and genus (D) level. Genera with a mean relative abundance value >0.1% are shown. The significance of differences among groups was determined using the metastats. All data are mean  $\pm$  standard error of mean.  $^{\#}P < 0.05$ ,  $^{##}P < 0.01$ , compared with PBS group;  $^{*}P < 0.05$ ,  $^{**}P < 0.01$ , compared with OVA group.  $n = 5$  per group. PBS: phosphate buffer saline; OVA: ovalbumin; AT: *Allium tuberosum*; OTU: operational taxonomic unit.

### 3.5. Bacterial biomarker

To further explore the role of gut flora in the treatment of AT, we performed linear discriminant analysis effect size analysis on bacterial sequences. As shown in Fig. 6A, the distribution histogram identified eight specific taxa that existed in the PBS group but not in the model or OVA + AT groups. The *Rikenellaceae\_RC9\_gut\_group* was the predominant colonic phylum. Three taxa were found in the OVA group, with *Romboutsia* and *Peptostreptococcaceae* showing co-dominance. Fifteen taxa were found in the OVA + AT group, and *Ruminococcaceae* were the predominant community member. The cladogram in Fig. 6B illustrates the relatedness of identified bacterial species, and shows remarkable differences at all levels. The branches for the *Ruminococcaceae*

and *Desulfovibrionaceae* were representative of the specific microbiota in the OVA + AT group.

## 4. Discussion

Our study shows that, in asthmatic mice, AT can alleviate asthma, change the structure of the gut microbiota and reduce the numbers of ILCs in both the lung and intestine. We also show that AT can reduce the serum concentration of IgE, thereby decreasing the IgE-mediated hypersensitivity. Further, AT enhanced eosinophils in BALF, increased infiltration of inflammatory cells in HE-stained lung tissue and increased mucus secretion in PAS-stained lungs.

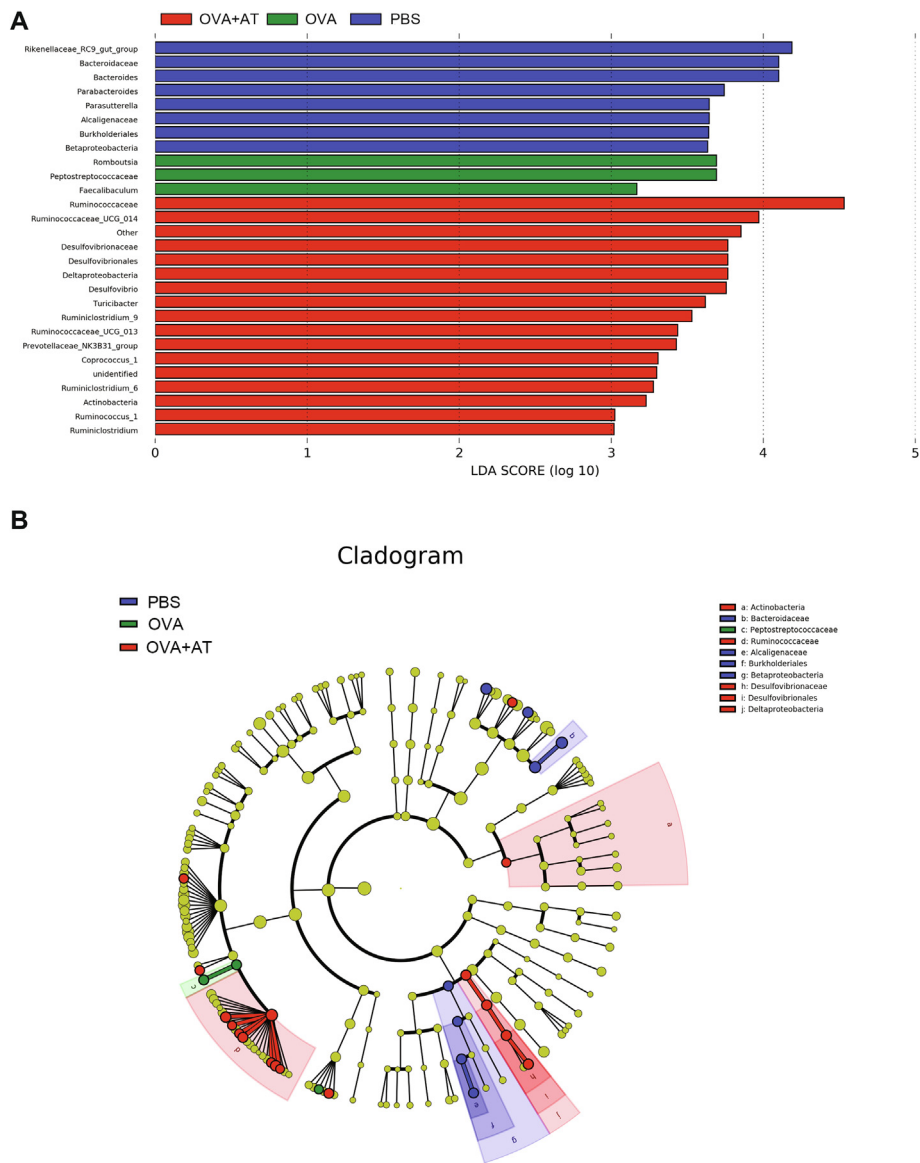


**Fig. 6.** Linear discriminant analysis effect size analysis of three groups. A: The histogram of the linear discriminant analysis score showed the biomarkers with the statistics difference of three groups. The influencing degree of species was expressed by the length of the bar in the histogram. B: In the cladogram, the circle radiated inside-out demonstrated the classification (from phylum to genus). Each small circle at different classification represented taxon and the diameter of the circle is proportional to the relative abundance. The species without significant differences were colored by yellow and biomarkers were colored by different groups. Red, blue and green dots represent the core bacterial populations in the respective group.  $n = 5$  per group. PBS: phosphate buffer saline; OVA: ovalbumin; AT: *Allium tuberosum*; LDA: linear discriminant analysis.

The immunological mechanisms of asthma are still not well understood, from the initial Th1/Th2 balance hypothesis to the later Th17/Treg balance hypothesis, to the recent discovery of ILC subpopulations [31]. It was shown in previous studies that ILCs were only a small fraction of innate immune cells in the lung but had a great influence on the lung [32]. IL-13, derived from ILC2, is critical for activating CD40<sup>+</sup> DC migration to drain lymph nodes, which subsequently induces adaptive Th2 immunity [33]. Therefore, researchers began to focus on ILCs linked to the development of asthma. ILC2 are dominant sources of ILCs in the lung at a steady state. ILC2 retains a degree of plasticity and can differentiate into ILC1 or ILC3, under certain environmental conditions. ILC2 is thought to be heterogeneous and at least has two populations, including ST2<sup>+</sup>KLRG1<sup>+</sup>ILC2 (nature ILC2, or nILC2) and ST2<sup>+</sup>KLRG1<sup>+</sup> ILC2 (inflammatory ILC2, or iILC2) [34]. nILC2 are the predominant population of ILC in the lung at a steady state, and they are responsive to IL-25, IL-33 and thymic stromal lymphopoietin (TSLP). KLRG1<sup>+</sup>ILC2 is barely expressed in the lung

under normal conditions, but it can respond more quickly to the stimulation of IL-25, while gaining the ability to produce IL-17, showing the characteristics of ILC3 [34]. And ST2<sup>+</sup> ILC2 is a critical mediator in the maintenance of airway hyperreactivity [35]. Our data indicate that there existed significantly increasing inclination of ILCs both from the lung and colon in OVA-induced asthmatic mice, while AT remarkably inhibited this enhancement. This phenomenon might be linked to a systemic reaction produced by chemokines in response to OVA aerosol inhalation. However, recently Huang et al. [29] found that KLRG1<sup>+</sup>ILC2 can migrate into both lymphatic and nonlymphatic organs in a partly S1P-dependent manner, participating in vital anti-helminth and tissue repair responses. Thus, it is possible that the population of ILCs in the colon of asthmatic mice originated in the lung; further studies are needed to explore this possible relationship. In fact, we also detected ST2<sup>+</sup>ILC2 in our experiment; however, differences among our study groups were not significant, possibly because of the small number of cells.

Next, we have demonstrated the effect of AT on the intestinal microbiota, although AT did not change the richness and diversification of gut microbes in asthmatic mice. *Ruminococcaceae* and *Desulfovibrionaceae* were identified as two biomarkers from the treatment effect of AT. We think there is excellent potential for research into the impact of these two bacteria on asthma. At present, more studies are investigating the roles of intestinal microbiota in asthma [36–38]. Some researchers have focused on the protective effects of probiotics on asthma and have carried out animal experiments to validate them. For example, the therapeutic effect of LGG on asthma [39] has been a great inspiration for clinical practice. However, *Desulfovibrionaceae* and *Ruminococcaceae* are currently less studied. The intake of beef products could cause the outgrowth of *Desulfovibrionaceae*, which can cause intestinal inflammation [40]. This is consistent with the increase in *Desulfovibrionaceae* we found in the OVA group. Some researchers have found that asthmatic children have increased levels of *Ruminococcaceae* [41]. However, West et al. [42] indicated that the lower relative abundance of *Ruminococcaceae* already existed at one week of age in infants who were developing IgE-associated eczema. At the genus level, the relative abundance of *Ruminococcus* was inversely associated with IL-6 and TNF- $\alpha$  induced by TLR-2. This might explain why the relative abundance of *Ruminococcaceae* became higher after AT treatment in our experiments. *Ruminococcaceae*, a member of short-chain fatty acid (SCFA) producers, is a family of autochthonous and benign species that contribute to the degradation of diverse polysaccharides and fibers. However, how these two taxa shape the development of asthma is not well understood. The intestines and lungs are the central locations of ILCs, and studies have found that ILCs take on the primary immune function of the gut before the adaptive immune system matures. After that, the system function of ILCs gradually weakens to balance the intestinal homeostasis [20]. This mechanism of ILCs is closely related to the colonization of the early neonatal microbiota. Whether *Desulfovibrionaceae* and *Ruminococcaceae* contribute to the differentiation and activation of the intestinal ILCs needs to be further studied.

## 5. Conclusion

This study focused on the effect of functional food on asthmatic mice and its mechanism of actions. We found that AT mitigated pulmonary inflammation, possibly by rectifying the disorder of gut microbiota and inhibiting the activation of ILC2. However, AT contains many chemical ingredients, and constituents responsible for its activity remain unknown. Future studies should focus on the identification of active components in AT and how the metabolites generated by the gut microbiota influence the immune responses of the lung and gut in asthmatic mice.

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## Author contributions

Hao-cheng Zheng: Conceptualization, Formal analysis, Methodology, Writing - original draft. Zi-ru Li: Conceptualization, Methodology. Ya-lan Li: Visualization. Yong-an Wang: Methodology. Jing-wei Kong: Formal analysis. Dong-yu Ge: Methodology. Gui-ying Peng: Funding acquisition, Project administration, Supervision, Writing - review & editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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